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The *URE2* Gene Product of *Saccharomyces cerevisiae* Plays an Important Role in the Cellular Response to the Nitrogen Source and Has Homology to Glutathione *S*-Transferases

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The *URE2* gene of *Saccharomyces cerevisiae* has been cloned and sequenced. It encodes a predicted polypeptide of 354 amino acids with a molecular weight of 40,226. Deletion of the first 63 amino acids does not have any effect on the function of the protein. Studies with disruption alleles of the *URE2* and *GLN3* genes showed that both genes regulate *GLN1* and *GDH2*, the structural genes for glutamine synthetase and NAD-linked glutamate dehydrogenase, respectively, at the transcriptional level, but expression of the regulatory genes does not appear to be regulated. Active *URE2* gene product was required for the inactivation of glutamine synthetase upon addition of glutamine to cells growing with glutamate as the source of nitrogen. The predicted *URE2* gene product has homology to glutathione *S*-transferases. The gene has been mapped to chromosome XIV, 5.9 map units from *petX* and 3.4 map units from *kex2*.

The yeast *Saccharomyces cerevisiae* has a number of proteins whose synthesis is regulated in response to the nitrogen source. The levels of the NAD-dependent glutamate dehydrogenase (NAD-GDH; the product of the *GDH2* gene [35]) and of glutamine synthetase (GS; the product of the *GLN1* gene [36]) are high in cells grown with glutamate as the nitrogen source but low in cells grown with glutamine as the nitrogen source. Both NAD-GDH and GS require glutamate for their reactions. NAD-GDH converts glutamate to α-ketoglutarate and ammonia, generating the ammonia for GS (35), while GS combines ammonia and glutamate to form glutamine. Glutamine is used for some biosynthetic reactions such as the production of certain amino acids and of nucleotides as well as for protein synthesis.

The regulation of *GLN1* and *GDH2* occurs in response to the nitrogen source at the level of transcription (1, 35). In addition, the GS enzyme may be inactivated by presenting glutamine to cells expressing GS. GS activity may be measured in two different ways, but both activities reside in the same molecule. The (biosynthetic) synthetase assay measures the activity of generating glutamine from glutamate and requires ATP (48, 50). This reaction is inactivated upon addition of glutamine to glutamate-grown cells (29, 39). GS inactivated in this manner may be reactivated by shifting the cells back to glutamate as the nitrogen source (29, 39). GS can also be measured by its ability to transfer the glutamate portion of glutamine to an arsenate molecule in the transferase assay (48, 50). This activity cannot be inactivated by addition of glutamine to glutamate-grown cells and has no known function (39).

Since NAD-GDH and GS are coordinately regulated, it is probable that there are genes whose products affect the regulation of both. Indeed, mutants have been isolated in which the regulation of both enzymes is altered, and these mutants fall into two complementation groups. Strains carrying mutations in the *GLN3* gene have low levels of NAD-GDH and GS activities when grown on either glutamate or glutamine as a nitrogen source (40). GS activity can also be increased by purine starvation or general amino acid starvation, and these systems were shown to be independent of *GLN3* (40). The strains carrying mutations in *GLN3* are also missing a number of other proteins, as determined by two-dimensional gel electrophoresis (40), as well as having decreased steady-state levels of mRNA transcribed from genes of the allantoin system (9).

Strains carrying mutations in the *URE2* gene have a phenotype opposite that of *gln3* strains. The levels of NAD-GDH, GS, and a number of other proteins are elevated in glutamate-grown cells, and for NAD-GDH this elevated level is even higher than that of wild-type cells grown on glutamate (10, 18). Alleles of *URE2* (also known as *usu* and *gdhCR*) (17, 18) have been isolated in a number of screens designed to isolate different mutants, such as those that cause increased activity of amino acid permeases (10), resistance of ureidosuccinate transport to nitrogen repression (13), and genetic derepression of NAD-GDH (18).

Some work has been done previously to elucidate the relationship between *URE2* and *GLN3*. Courchesne and Magasanik have shown that *gln3* is epistatic to *ure2* (10). The *GLN3* gene has been cloned in our laboratory (35a). We report here the cloning of *URE2*. We also report on analysis of the effect of disruptions of *GLN3* and *URE2* on NAD-GDH and GS activity. Sequence analysis shows that the predicted *URE2* gene product has homology to glutathione *S*-transferases, and this observation suggests possible modes of regulation, which are discussed.

MATERIALS AND METHODS

Strains. The strains used are listed in Table 1. Strains with designations beginning with P, PH, and PD have similar backgrounds. They are approximately one-fourth Belgian (derived from Σ1278b) and three-fourths American (derived from S288C). Mapping strains were obtained from G. Fink and C. Martin.

Media. Media were prepared as described by Mitchell and Magasanik (39, 40) and Sherman et al. (51). Glucose and raffinose were used as sources of carbon and energy at a final concentration of 2%.

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TABLE 1. Strains

Strain	Genotype	Source[a]
S. cerevisiae		
P7-6A	*MATa ade2-102 leu2-3,112 ure2-1 GLN1-lacZ*	
P5-5D	*MATa ade2-102 ura3-52 ure2-1*	
PM43	*MATa ade2-102 leu2-3,112 ura3-52 gdh1-6 gln3Δ4::LEU2*	P. Minehart
P24-000-B-4C	*MATα leu2-3,112 his4-619 ure2Δ11::LEU2*	
DBY745	*MATα ade1-100 leu2-3,112 ura3-52*	D. Botstein
PM38	*MATα leu2-3,112 ura3-52*	P. Minehart
PH2	*MATα leu2-3,112 ura3-52 ure2Δ12::URA3*	
PM71	*MATα leu2-3,112 ura3-52 gln3Δ5::LEU2*	P. Minehart
F571	*MATa his2 leu1 lys1 met4 pet8 spo11 ura3*	G. Fink
L1456	*MATa arg8 leu2-3 petX pha2 prt1*	G. Fink
Z182	*MATa his3-Δ200 ade2 leu2-3,112 ura3-52 srb1Δ1::LEU2(=kex2)*	C. Martin (33)
P40-1A	*MATα leu2-3,112 his4-619 ade2-102 gln3Δ4::LEU2 ure2Δ11::LEU2*	
P40-1B	*MATa leu2-3,112 ura3-52 his4-619*	
P40-1D	*MATα leu2-3,112 ade2-102 gdh1-6*	
P40-2A	*MATα leu2-3,112 his4-619 ure2Δ11::LEU2*	
P40-2C	*MATa leu2-3,112 ura3-52 ade2-102 gln3Δ4::LEU2*	
P40-3A	*MATa leu2-3,112 his4-619 gln3Δ4::LEU2 ure2Δ11::LEU2*	
P40-3C	*MATα leu2-3,112 ura3-52 ade2-102 ure2Δ11::LEU2*	
P40-5B	*MATa leu2-3,112 ura3-52 ade2-102 ure2Δ11::LEU2*	
P40-5C	*MATα leu2-3,112 his4-619 gln3Δ4::LEU2*	
P40-5D	*MATα leu2-3,112 ura3-52 ade2-102 gln3Δ4::LEU2 ure2Δ11::LEU2*	
P40-6A	*MATa leu2-3,112*	
PD1	P40-1B × P40-1D	
PD2	P40-6A × P40-5C	
PD3	P40-2C × P40-2A	
PD4	P40-3A × P40-3C	
PD5	P40-2C × P40-5C	
PD6	P40-2C × P40-1A	
PD7	P40-3A × P40-5D	
PD8	P40-6A × P40-2A	
PD9	P40-5B × P40-2A	
E. coli		
HB101	F^- *hsdS20* (r_B^- m_B^-) *supE44 ara-14 galK2 lacY1 proA2 rspL20*(Sm^r) *xyl-5 mtl-1 recA13*	4
XL1-Blue	*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1 (F' proAB lacI^q lacZΔM15* Tn*10* (Tet^r)	Stratagene
GW3810	*supE thi sbcB rpsL*(Sm^r) *endA1 hsdR4 Δ(lac-proAB) dam*::Tn*9*(Cam^r) (F' *traD36 proAB lacI^q lacZΔM15*	G. Walker

[a] Strains that have no source listed are from this study.

Genetics. Standard yeast genetic methods were used (51). Map units were calculated as described by Perkins (45).

Assays. Yeast cells were grown to a density of 80 to 120 Klett units and then harvested as described by Mitchell and Magasanik (39). Cell extracts were made as described by Mitchell and Ludmerer (37) and used immediately.

Protein concentrations were determined as described by Bradford (5), using reagents obtained from Bio-Rad Laboratories (Richmond, Calif.). GS synthetase and transferase activities were assayed as described by Mitchell and Magasanik (38).

NAD-GDH assays were performed as described by Miller and Magasanik (35). Some variability is normally seen when the assays are performed on different days. To reduce this variability for each table, cultures were grown and assayed together.

Assays for β-galactosidase were performed by using crude cell extracts as described by Miller (34).

DNA preparations. Yeast plasmids were prepared essentially as described by Sherman et al. (51) except that the volume of cells used was 10 ml. Bacterial plasmids were prepared by the boiling method of Holmes and Quigley (23). The alkaline lysis method of Maniatis et al. (32) was used for large-scale CsCl bacterial plasmid preparations.

Plasmid constructions. DNA manipulations were carried out as described by Maniatis et al. (32). Enzymes were used as instructed by the manufacturer's (generally New England BioLabs [Beverly, Mass.] for restriction enzymes, ligase, and Klenow enzyme and Boehringer Mannheim [Indianapolis, Ind.] for calf alkaline phosphatase).

To create plasmid p1-XS, the *Xba*I-*Sal*I fragment of the original clone of *URE2* was ligated into the *Nhe*I-*Sal*I site of YEp24 (3). Plasmid p1XSΔSac is a *Sac*II deletion of this plasmid. Plasmid p1C-CS was constructed by inserting the *Cla*I-*Sal*I fragment of p1-XS into the *Cla*I-*Sal*I site of YCp50 (26). Plasmids p1C-RE, p1C-CSΔH, and p1C-CSΔU are deletion constructions of p1C-CS. Plasmid p1I-BS was created by isolating the *Bgl*II-*Sal*I fragment from p1-XS and inserting it into the *Bam*HI-*Sal*I site of YIp5 (53).

The first null allele of *URE2* was constructed by replacing the *Sac*II-*Pvu*II portion of the coding region with the *Sal*I-*Xho*I fragment of YEp13 (6) containing *LEU2*. The second null allele was created by replacing the *Hin*dIII fragment of coding and upstream DNA with the *Hin*dIII fragment of YEp24 containing the *URA3* gene. In each case, the chromosomal copy of *URE2* was then disrupted as described by Rothstein (47).

Plasmid p9, carrying the *URE2-lacZ* fusion, was constructed by isolating the *Sal*I-*Eag*I fragment of *URE2* and filling the ends with Klenow enzyme. Plasmid pLGΔ312 (20),

a 2μm vector, was cut with *Sma*I and *Bam*HI and also filled in with Klenow enzyme. The two fragments were ligated together, resulting in a plasmid that codes for a fusion protein containing the first 65 amino acids of URE2. The junction was sequenced to confirm the construct. Control plasmid pSLFΔ178K (15) was derived from pLGΔ312 by deletion of the *Sma*I-*Xho*I fragment that contains the upstream activation sequences of *CYC1*. Plasmid pPM46, carrying the *GLN3-lacZ* fusion (35a), was also derived from pLGΔ312.

Plasmid p7LU, carrying the *GAL10-URE2* construct, which contains the transcriptional start site from the *GAL10* gene and translational start from the *URE2* gene, was made by inserting the 1.2-kb *Taq*I-*Cla*I fragment (the *Taq*I site used is underlined with a wavy line in Fig. 5) containing the truncated *URE2* gene into the *Sma*I site of plasmid pKP15, a derivative of pLGSD5 (21), obtained from Karl Pfeiffer, and then inserting a *Sal*I-*Xho*I fragment containing the *LEU2* gene into the *Sal*I site. The truncated *URE2* is now expressed when cells are grown on raffinose and can be greatly induced by adding galactose (2% final concentration) to the culture. Other plasmids used include Bluescript (Stratagene, La Jolla, Calif.) and pJT71 (the source of the *TUB2* fragment, kindly supplied by Jim Thomas).

Transformations. Yeast transformations were carried out either as described by Ito et al. (24) or as described by Hinnen et al. (22). Directed integration of plasmids into the yeast chromosome was performed by cutting plasmid p1I-BS carrying *URE2* with *Xho*I as described by Orr-Weaver et al. (42). Chromosomal disruptions were made as described by Rothstein (47) by using a *Eag*I-*Cla*I partial digest fragment for *ure2Δ11::LEU2* and a *Sal*I-*Cla*I fragment for *ure2Δ12::URA3*. Gapped plasmid repair was carried out as described by Orr-Weaver et al. (43). Bacterial transformations were done as described by Maniatis et al. (32).

Chromosomal mapping. The blot of chromosomal DNA separated by pulse field electrophoresis (Clontech, Palo Alto, Calif.) was probed with the 0.85-kb *Sac*II-*Eag*I fragment of *URE2* or the 3.6-kb *Hin*dIII fragment of *KEX2* (obtained from Chris Martin) as described by Eisenmann et al. (14).

Preparation and analysis of RNA. Total yeast RNA was purified essentially as described by Carlson and Botstein (7). Poly(A)$^+$ RNA was then prepared in batch as described by Maniatis et al. (32). RNA was separated on a 1% agarose formaldehyde gel (30) and transferred to GeneScreen (NEN DuPont, Boston, Mass.) according to the manufacturer's instructions except that the RNA was blotted with 1× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and fixed with UV irradiation. ^{32}P-labeled probes (2.6-kb *Sal*I fragment for *GDH2* [35] and 0.9-kb *Sal*I-*Kpn*I fragment for *TUB2*) were made with a nick translation kit as instructed by the manufacturer (Boehringer Mannheim) (46), and hybridization was carried out as described by Clark-Adams and Winston (8).

DNA sequencing. DNA was sequenced by the dideoxy method of Sanger et al. (49), using [α-^{35}S]dATP. Sequenase enzyme (modified T7 polymerase) and reagents were obtained in a Sequenase kit from U.S. Biochemicals (Cleveland, Ohio). The Bluescript vector and most primers were obtained from Stratagene.

Computer methods. Homology searches were carried out with the FASTA program (44) against a data base which contains NBRF (Georgetown University Medical Center, Washington, D.C.) protein residues (release 23.0, December 1989) and the translation of GenBank (Los Alamos National Laboratories, Los Alamos, N.M.) nucleic acid sequences (release 63.0, April 1990). The BESTFIT (52) and LINEUP programs in the GCG package (version 6.1, August 1989) (University of Wisconsin, Madison) (12) were used for homology and sequence comparisons.

Nucleotide sequence accession number. The sequence reported here has been deposited in the GenBank data base and has given accession number M35268.

RESULTS

Cloning of *URE2*. *URE2* regulates a *GLN1-lacZ* fusion (1) in the same manner as it regulates *GLN1* and *GDH2*. In a wild-type background, the β-galactosidase activity is high when glutamate is the nitrogen source and low when glutamine is the nitrogen source. It is always high, irrespective of the nitrogen source, in a *ure2* strain. To clone *URE2*, strain P7-6A (which carries *ure2-1* and the *GLN1-lacZ* fusion) was transformed with a YEp13-derived yeast clone bank (kindly supplied by Peter Drain), and colonies were selected for their ability to grow in the absence of leucine. Six candidate transformants of about 6,000 screened appeared to be complemented for *ure2-1* on glucose glutamine 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) plates by showing the reduced level of β-galactosidase expected of wild-type cells grown on glutamine. These six strains were grown on glutamine and assayed for GS and NAD-GDH activities. Only two had low wild-type levels of activity in addition to the reduced level of β-galactosidase. The complementing plasmids were isolated from these two strains and passaged through *Escherichia coli*. Preliminary restriction analysis indicated that the clones overlapped; therefore, further analysis was continued with only one clone.

Figure 1A shows the restriction map of the insert carried by complementing plasmid and the map of several subclones that were constructed. The subclone's ability to complement the *ure2-1* allele is indicated in Fig. 1B. A strain carrying *URE2* on a high-copy-number plasmid had slightly reduced levels of NAD-GDH activity. The *Eag*I-*Eco*RI fragment was sufficient to complement the *ure2-1* mutation. Additional analysis of the size of *URE2* will be discussed below.

To confirm that the cloned gene was indeed *URE2*, plasmid p1I-BS (see Materials and Methods) was linearized with *Xho*I and integrated into P5-5D (*ure2-1 ura3-52*) at the site of homology (42). The resultant transformant was crossed to a wild-type strain (DBY745), and the diploid was sporulated and dissected. All 37 tetrads examined gave rise to 4 *URE2*:0 *ure2* spores, indicating that the cloned gene is almost certainly *URE2* since it is located less than 1.4 map units from *URE2*.

Two disruptions of *URE2* were also made (Fig. 1A; for details of the constructions, see Materials and Methods). The first disruption was created in a diploid strain to test the potential lethality of the *ure2* deletion. Sporulation and dissection of tetrads gave rise to four viable colonies, indicating that *URE2* is a nonessential gene. A DNA blot of this disrupted strain gave a pattern expected of a *URE2* disruption (data not shown). Both disruptions have phenotypes indistinguishable from that of a *ure2-1* strain: high levels of GS and NAD-GDH when grown with either glutamate or glutamine as a nitrogen source as well as much slower growth rates than wild type. Contrary to wild-type strains, the growth rate of a *ure2* disruption strain is slightly higher with glutamate as a source of nitrogen than it is with glutamine.

Mapping of *URE2*. The *URE2* gene was localized to

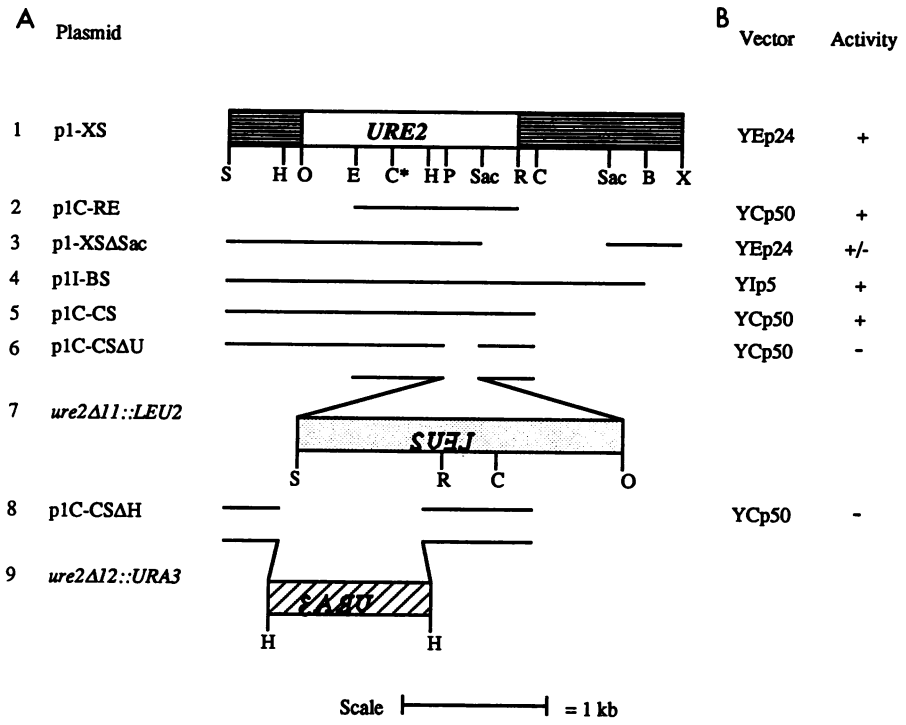

FIG. 1. Restriction map of the *URE2* gene. Restriction sites are labeled as follows: B, *Bgl*II; C, *Cla*I; C*, *Cla*I blocked by *dam* methylation; E, *Eag*I; H, *Hin*dIII; O, *Xho*I; P, *Pvu*II; R, *Eco*RI; S, *Sal*I; Sac, *Sac*II; X, *Xba*I. *ure2Δ11::LEU2* was created by inserting the *LEU2* gene into p1C-CSΔU in the orientation opposite that of *URE2*. Only the *Xho*I site of the *LEU2* fragment was recreated in this construct. *ure2Δ12::URA3* was created by inserting the *URA3* gene into p1C-CSΔH in the orientation opposite that of *URE2*.

chromosome XIV by probing a blot of pulse field-separated yeast chromosomal DNA (purchased from Clontech, Palo Alto, Calif.) with a labeled fragment of *URE2* DNA and confirmed by reprobing the same stripped blot with a known chromosome XIV gene (a *KEX2* fragment) (data not shown). The map position was then determined by crossing *ure2* marked strains to a variety of strains carrying chromosome XIV mutations and analyzing tetrads. The results for some of these crosses (Table 2) show that *URE2* can be placed on the genetic map between *kex2* and *petX*, 3.4 map units from *kex2* and 5.9 map units from *petX*.

Effect of disruptions of *GLN3* and *URE2*. We determined the levels of NAD-GDH and of GS in haploid wild-type cells and in cells in which *GLN3*, *URE2*, or both had been disrupted. All cells were grown with either glutamate or glutamine as the source of nitrogen (Table 3). The results of these experiments confirmed previously published findings using point mutations in *GLN3* (nonsense) and *URE2* (missense) (10). In the wild-type strain, the levels of both enzymes were much higher in glutamate-grown cells than in glutamine-grown cells (Table 3). In strains with *GLN3* disrupted, the levels of both enzymes were low in glutamate- and in glutamine-grown cells, irrespective of the state of the *URE2* gene. In the strain with functional *GLN3* but with *URE2* disrupted, the levels of both enzymes in glutamine-grown cells were almost as high as their levels in glutamate-grown cells. In cells grown on glutamate, the disruption of *URE2* resulted in an approximately sixfold increase in the level of NAD-GDH but in a very small increase in the level of GS.

The results of the experiment with diploids carrying different dosages of functional *GLN3* and *URE2* confirmed that the great increase in the levels of both NAD-GDH and GS in glutamate-grown cells depends on a functional *GLN3* gene, irrespective of the state of *URE2* (Table 4, experiments 1 to 3). Furthermore, they show that a single copy of *URE2* was as effective as two copies of *URE2* in reducing the levels of both enzymes in glutamine-grown cells as well as in reducing the level of NAD-GDH in glutamate-grown cells (Table 4, experiments 4 to 9). However, the level of NAD-GDH, but not of GS, was strongly affected by the dosage of *GLN3*. The level of NAD-GDH in glutamate-grown diploid cells with two copies of *GLN3* and either one or two copies of *URE2* corresponded to the level of NAD-GDH in glutamate-grown haploid cells of the wild type (Table 4, experiments 5 and 6; Table 3, experiment 3). On the other hand, in glutamate-grown cells with a single copy of *GLN3* and either one or two copies of *URE2*, the level of NAD-GDH was only one-half

TABLE 2. Mapping of *ure2*

Interval	Tetrads[a]				Calculated map distance[b]
	Total	PD	NPD	TT	
ure2-met4	28	6	2	20	57.1[c]
ure2-pet8	28	3	4	21	Unlinked[c]
ure2-petX	51	45	0	6	5.9[d]
ure2-pha2	21	1	2	18	Unlinked[d]
ure2-kex2	44	41	0	3	3.4[e]

[a] PD, Parental ditype; NPD, nonparental ditype; TT, tetratype.
[b] Expressed in centimorgans and calculated according to the formula of Perkins (45).
[c] Determined from cross PH2 × F571.
[d] Determined from cross P40-2A × L1456.
[e] Determined from cross PH2 × Z182.

TABLE 3. Effect of *GLN3* and *URE2* on NAD-GDH and GS activities in haploid cells[a]

Expt	Strain	Genotype[b]		Sp act			
				NAD-GDH (nmol min^{-1} mg of $protein^{-1}$)		GS (μmol min^{-1} mg of $protein^{-1}$)	
		GLN3	*URE2*	glt	gln	glt	gln
1	P40-1A	−	−	3.3 ± 0.2	2.5 ± 0.7	0.10 ± 0.01	0.02 ± 0.01
2	P40-2C	−	+	4.9 ± 0.5	1.7 ± 0.8	0.11 ± 0.01	0.03 ± 0.01
3	P40-1B	+	+	26.8 ± 1.2	2.2 ± 0.6	1.03 ± 0.03	0.03 ± 0.01
4	P40-3C	+	−	169.5 ± 1.6	125.3 ± 2.0	1.35 ± 0.04	0.82 ± 0.02

[a] Cells were grown with glutamate (glt) or glutamine (gln) as a source of nitrogen and with adenine, leucine, histidine, and uracil to supplement the auxotrophies of some of the strains.
[b] +, Wild-type allele; −, disrupted allele.

that found in corresponding cells of the haploid wild type (Table 4, experiments 8 and 9; Table 3, experiment 3). This gene dosage effect of *GLN3* was also apparent in diploids lacking functional *URE2* when these cells were grown on glutamate and became even more striking when these cells were grown on glutamine. The level of NAD-GDH was 25% lower in glutamine-grown cells of both the diploid with two copies of *GLN3* and no copies of *URE2* and the haploid with one copy of *GLN3* and no copies of *URE2* than it was in corresponding glutamate-grown cells (109.6 versus 148.8 for the diploid PD9 [Table 4, experiment 4] and 125.3 versus 169.5 for the haploid P40-3C [Table 3, experiment 4]), but this level was 70% lower in corresponding diploid cells with a single copy of *GLN3* (26.6 versus 88.4 for the diploid PD4 [Table 4, experiment 7] when grown on glutamine). This effect of glutamine, previously observed in an experiment using a diploid carrying a single functional *GLN3* gene and the mutant genes *gln3-1* and *ure2-1* (9a), was shown to result from a decrease in the abundance of the mRNA encoded by *GDH2*, the structural gene of NAD-GDH (Fig. 2).

Expression of *URE2*. To address the question of abundance of the *URE2* and *GLN3* gene products in the cell, strain PM38 was transformed with plasmid pSLFΔ178K (15), pPM46 (a *GLN3-lacZ* fusion plasmid; 35a), or p9 (a *URE2-lacZ* fusion plasmid; for details of the construction, see Materials and Methods). These strains were grown with either glutamate or glutamine as a source of nitrogen, harvested, and assayed for β-galactosidase activity. Neither *GLN3-lacZ* nor *URE2-lacZ* was regulated at the level of transcription or of initiation of translation by the nitrogen source (Table 5). This finding confirms the result in our laboratory (35a) that the chromosomal *GLN3* gene is not regulated at the level of transcription or initiation of translation (regardless of the state of the *URE2* gene). It is also consistent with the observation that *URE2* is not regulated at the level of transcription or protein abundance (unpublished results).

There was about four times more *GLN3-lacZ* product than *URE2-lacZ* product (Table 5). This result is consistent with the idea that the *URE2* gene product acts catalytically on the *GLN3* gene product to regulate transcription of *GLN1* and *GDH2* (see Discussion). This view agrees with the observation (see above) that changes in the amount of the *GLN3* gene product present can drastically affect the expression of *GDH2*, while similar changes in the amount of *URE2* gene product have only a slight effect.

Inactivation of GS. It had previously been shown that the addition of glutamine to glutamate-grown cells inactivated the synthetase activity of GS without significantly affecting the transferase activity (39). It has also been reported that a mutation in *URE2* prevented this inactivation (29). We further analyzed this effect by using a disruption of *URE2*. In a wild-type strain, GS synthetase activity was rapidly inactivated upon addition of glutamine to a glutamate-grown culture, while GS transferase activity was unaffected (Fig. 3). In strains that carry either the *ure2-1* allele or the *ure2Δ12::URA3* disruption, this inactivation of GS synthetase activity did not occur. Provision of a raffinose-grown *ure2*-disrupted strain with a plasmid encoding the truncated URE2 protein (see below) behind the *GAL10* promoter

TABLE 4. Gene dosage effect of *GLN3* and *URE2* on NAD-GDH and GS activities[a]

Expt	Strain	Genotype[b]		Sp act			
				NAD-GDH (nmol min^{-1} mg of $protein^{-1}$)		GS (μmol min^{-1} mg of $protein^{-1}$)	
		GLN3	*URE2*	glt	gln	glt	gln
1	PD7	−/−	−/−	3.1 ± 0.2	1.0 ± 0.1	0.08 ± 0.01	0.01 ± 0.01
2	PD6	−/−	−/+	5.8 ± 0.3	1.0 ± 0.1	0.11 ± 0.01	0.02 ± 0.01
3	PD5	−/−	+/+	3.8 ± 0.2	1.1 ± 0.1	0.11 ± 0.02	0.01 ± 0.01
4	PD9	+/+	−/−	148.8 ± 4.8	109.6 ± 3.7	1.42 ± 0.04	1.40 ± 0.04
5	PD8	+/+	−/+	25.3 ± 1.9	0.7 ± 0.1	1.06 ± 0.01	0.03 ± 0.01
6	PD1	+/+	+/+	28.1 ± 0.5	0.5 ± 0.1	1.42 ± 0.04	0.01 ± 0.01
7	PD4	−/+	−/−	88.4 ± 3.8	26.6 ± 0.2	0.94 ± 0.22	0.67 ± 0.03
8	PD3	−/+	−/+	9.4 ± 1.4	1.1 ± 0.1	1.09 ± 0.04	0.02 ± 0.01
9	PD2	−/+	+/+	10.6 ± 0.4	1.1 ± 0.3	0.93 ± 0.06	0.03 ± 0.01

[a] Cells were grown with glutamate (glt) or glutamine (gln) as a source of nitrogen and with adenine, leucine, histidine, and uracil to supplement the auxotrophies of some of the strains.
[b] +, Wild-type allele; −, disrupted allele.

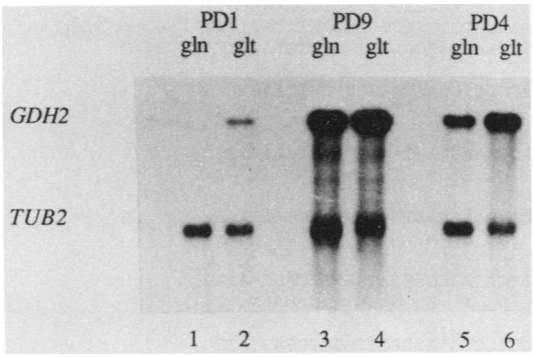

FIG. 2. Northern (RNA) analysis of diploid strains. Cells were grown on either glutamine (gln) or glutamate (glt) as a nitrogen source, as indicated at the top of each lane. Lanes: 1 and 2, strain PD1 (*GLN3/GLN3 URE2/URE2*); 3 and 4, strain PD9 (*GLN3/GLN3 ure2Δ11::LEU2/ure2Δ11::LEU2*); 5 and 6, strain PD4 (*GLN3/gln3Δ4::LEU2 ure2Δ11::LEU2/ure2Δ11::LEU2*). Approximately equal amounts of poly(A)$^+$ RNA were loaded into each lane. The blot was probed with both a *GDH2* fragment and a *TUB2* fragment (to standardize the amount of RNA loaded) as explained in Materials and Methods.

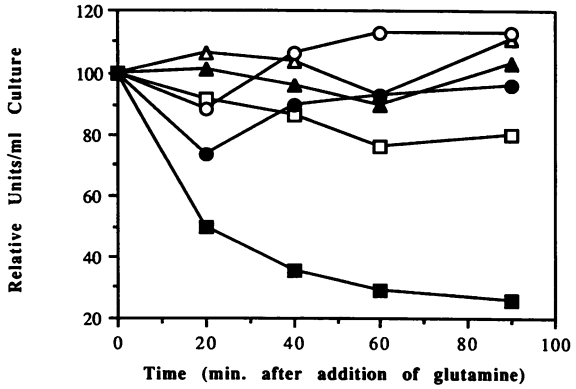

FIG. 3. Analysis of inactivation of GS in vivo. Strains PM38 (wild type) (□, ■), PH2 (*ure2Δ12::URA3*) (△, ▲), and P5-5D (*ure2-1*) (○, ●) were grown in glucose glutamate to about 70 Klett units, and then glutamine was added to the culture to a final concentration of 0.2%. At the indicated time points, equal volumes of liquid were removed from the flask, and the cells were collected and frozen. GS transferase assays (open symbols) and synthetase assays (filled symbols) were performed as described by Mitchell and Magasanik (38). Relative units per milliliter of culture equals the specific activity times the Klett units. These values were then normalized to a value of 100 at time = 0.

allowed the GS synthetase activity to be inactivated (Fig. 4). The amino-terminal portion of the protein is therefore not required for inactivation of GS synthetase activity.

Cells carrying the *gln3* mutation can be partially induced for GS by adenine starvation (41). Such a strain (PM71, which carries *gln3Δ5::LEU2*) was also examined for inactivation of GS synthetase activity. The low amount of activity in adenine-starved cells grown with glutamate as a source of nitrogen was rapidly reduced to the basal level upon addition of glutamine to the medium (data not shown), indicating that the *GLN3* gene product is not needed for the inactivation of GS.

In view of the fact that the *URE2* product is required for both the inactivation of GS and the reduction in the expression of *GLN1* and *GDH2*, the structural genes for GS and NAD-GDH, respectively, we considered the possibility that the effect on gene expression is mediated through GS. For example, active GS could be required for activation of expression of *GDH2*, or the inactivated GS could block the activation of the expression of *GLN1* and *GDH2*. We examined these possibilities by measuring the level of NAD-GDH in glutamine-grown cells of strains mutated in *GLN1* (a nonsense allele), *URE2*, both *GLN1* and *URE2*, or neither. The level of this enzyme was as high in cells mutated in both *GLN1* and *URE2* as it was in corresponding cells of a strain lacking only *URE2* (data not shown), showing that active GS is not required to activate expression of *GDH2*. The level of NAD-GDH in a strain mutated in the *GLN1* gene grown on glutamine was as low as that in a wild-type strain grown under the same conditions, showing that the presence of inactive GS is not required to block the activation of expression of *GDH2* (data not shown). These results indicate that glutamine synthetase is not involved in the effects of the *URE2* gene product on the expression of *GDH2* and, presumably, *GLN1*.

Sequence of *URE2*. The sequence of the *URE2* gene from the *Xho*I site to the *Eco*RI site was determined by the method of Sanger et al. (49) as explained in Materials and Methods. The entire 1,427-bp sequence is shown in Fig. 5.

TABLE 5. Translational regulation of *URE2* and *GLN3*[a]

Expt	Plasmid[b]	β-Galactosidase (U)[c]	
		glt	gln
1	pSLFΔ178K	2 ± 1	4 ± 1
2	pPM46	220 ± 6	174 ± 6
3	p9	47 ± 1	54 ± 1

[a] Strain PM38 (*MATaleu2-3,112 ura3-52*) was transformed with each of the indicated plasmids and grown with either glutamate (glt) or glutamine (gln) as a source of nitrogen.

[b] Plasmids pSLFΔ178K is a control plasmid that has an ATG but no upstream activation sequences (15), pPM46 carries the *GLN3-lacZ* construct (35a), and p9 carries the *URE2-lacZ* construct.

[c] Calculated as described by Miller (34).

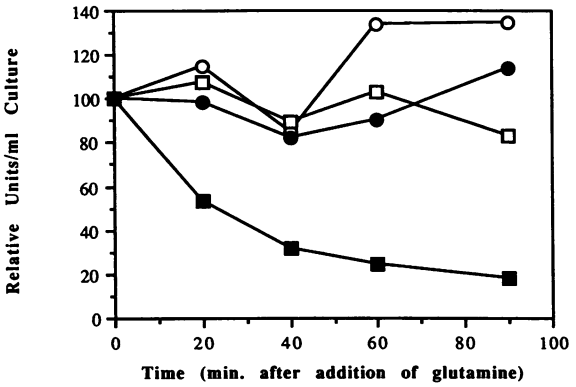

FIG. 4. Complementation by truncated *URE2* of the *ure2Δ12::URA3* disruption. Strain PH2 carrying either pSB32 (a *LEU2* control plasmid obtained from Joshua Trueheart) (○, ●) or p7LU (a *LEU2* plasmid carrying the truncated *URE2* behind the GAL10 promoter) (□, ■) was grown and assayed as described in the legend to Fig. 3 except that raffinose was the source of carbon and energy. GS transferase assays are indicated by the open symbols; synthetase assays are indicated by the filled symbols.

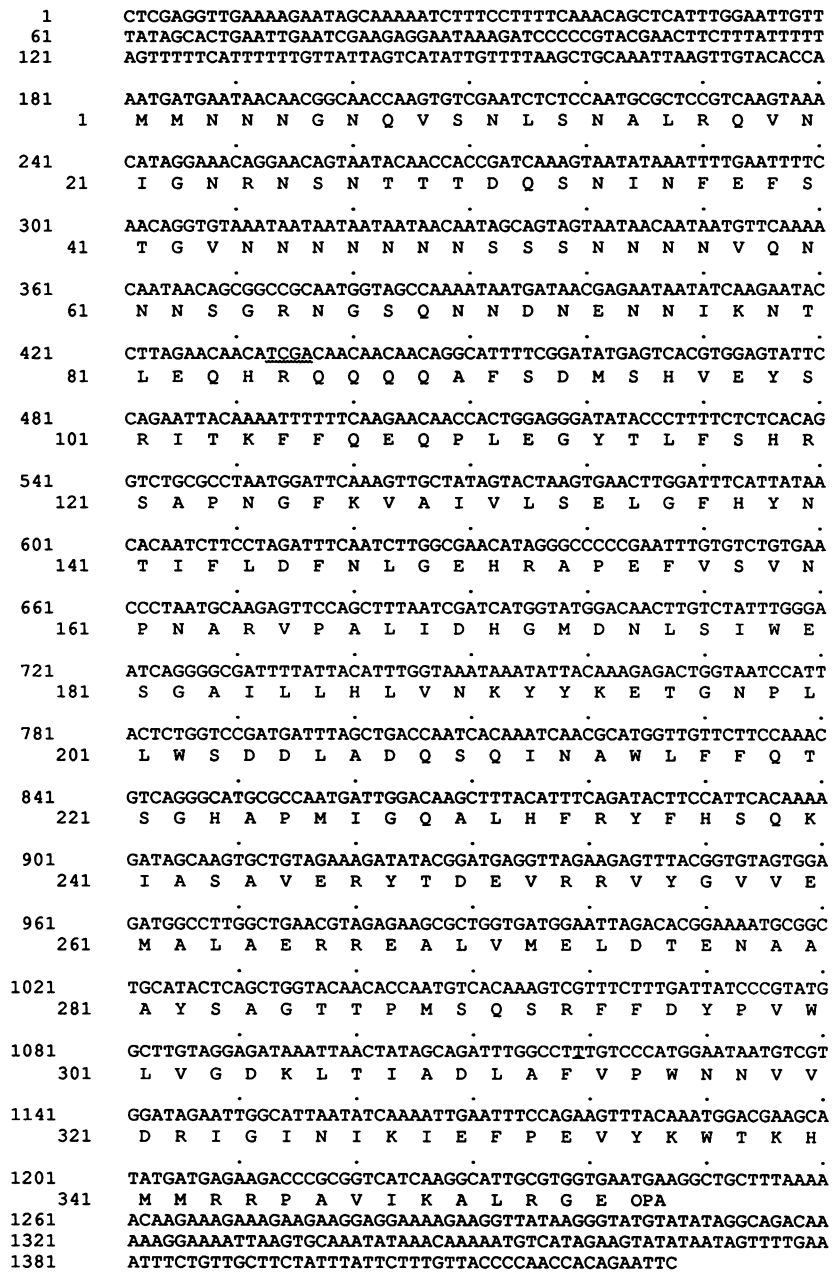

FIG. 5. Sequence of the *URE2* gene. The nucleotide sequence from the *Xho*I site to the *Eco*RI site is given. The predicted amino acid sequence is also included. The T at bp 1119 is underlined. This T is changed to a C in the *ure2-1* mutation.

An open reading frame of 1,062 bp begins at base 182. This sequence translates to a polypeptide of 354 amino acids with a predicted molecular weight of 40,226 whose sequence is also included in the figure.

Examination of the protein sequence shows that it contains no cysteine residues. However, there is an abundance of asparagine and serine residues, particularly in the amino-terminal portion. These observations indicate that the amino-terminal portion of the polypeptide is highly polar.

An unusual observation of the cloning and sequence analysis was that in certain contexts a fragment smaller than the entire gene could fully complement the *ure2-1* and disruption alleles. For instance, *URE2* constructs deleted to the *Eag*I site (to bp 370 in Fig. 5) and placed behind the *GAL10* promoter (such that *GAL10* supplies the start of transcription while the first ATG codon after the TATA box is contained in the *URE2* fragment) could fully complement *ure2Δ12*::*URA3* (in which the *Hin*dIII-*Hin*dIII segment is deleted). This same fragment placed in the *Eag*I site within the *tet* gene of YCp50 (p1C-RE) also complemented *ure2-1*. However, when this fragment was reversed in orientation and moved to the *Bam*HI site within the *tet* gene, it failed to complement. A possible explanation for the varying complementation results is that a cryptic yeast promoter is present in the *tet* gene which allows expression of the *URE2* fragment when the gene is placed nearby. This suggests that the

shortened gene can complement only when sufficiently expressed and that the amino-terminal portion of the protein is not essential for any assayable function under these conditions.

Identification of the *ure2-1* mutation. To locate and identify the *ure2-1* mutation, the technique of gap plasmid repair was used to rescue it from the chromosome of a mutant strain (43). Plasmids were systematically deleted of portions of *URE2*, and the linearized DNA was transformed into the *ure2-1* strain, P5-5D. These linear plasmids were repaired via gene conversion, using the chromosomal copy as the template. If the corresponding DNA used for repair contained the mutation, then only *ure2* colonies would result. Deletion and repair of the *Sac*II-*Pvu*II fragment gave rise to *ure2* colonies; therefore, this plasmid was isolated and transformed into bacteria for sequence analysis. The alteration was found to be T-to-C transition (underlined in Fig. 5) at base 1119 causing the phenylalanine at amino acid 313 to be replaced by serine. Thus, the replacement of a hydrophobic, aromatic amino acid by a polar amino acid can disrupt the function of the protein, possibly by altering its structure.

Homology. The sequence of the predicted URE2 polypeptide was used to search for homology by computer analysis. Moderately strong homology scores were obtained to maize glutathione *S*-transferase III (19) and *Drosophila* glutathione *S*-transferase 1-1 (54), while weaker homology scores were obtained to human glutathione *S*-transferase 2 (2) and rat glutathione *S*-transferase subunit Y_a (27). A weak match was also obtained to *dcmA*, a gene coding for dichloromethane dehalogenase from *Methylobacterium* sp. strain DM4 (28). The product of this gene is an enzyme that requires glutathione as a cofactor.

All of these sequences except that for rat glutathione *S*-transferase are shown in Fig. 6. There are two regions that appear to be highly conserved in all of the proteins. The first of these regions (from about URE2 amino acids 178 to 192) has been proposed to be involved in the binding of glutathione because of its conservation among many glutathione *S*-transferases and because of the presence of what is thought to be an essential arginine residue (25). URE2 does not have an arginine in this area. The second region (from URE2 amino acids 300 to 314) is also conserved in glutathione *S*-transferases, but its function is unknown. The phenylalanine that is mutated in the *ure2-1* strain is in this second region of homology (at amino acid 313). It is tempting to speculate that this second region is crucial to URE2 protein function and that changing its composition away from the consensus by introducing a polar residue at the edge destroys protein function and possibly structure.

DISCUSSION

Our results show that the product of the *URE2* gene is required for the inactivation of GS by glutamine and plays a major part in the repression of *GLN1* and *GDH2* by glutamine. Disruption alleles of *URE2* have the same phenotype as the *ure2-1* missense allele, indicating that *ure2-1* does not produce a partially functional URE2 protein. Sequencing of *URE2* has shown that its product is a polypeptide of 354 amino acid residues. The 63 amino acid residues at the amino-terminal end are not required for the function of the protein. Deletion of the portion of the gene coding for these amino acid residues results in a truncated protein still able to bring about inactivation of GS and repression of *GLN1* and *GDH2*.

The carboxyl-terminal portion of the *URE2* gene product, which is responsible for its activity, shows considerable homology to glutathione *S*-transferases of maize (19), *Drosophila* (54), rat (27), and human (2) cells. The *ure2-1* mutation falls in a region of high homology and inactivates the *URE2* gene product. It is therefore possible that the *URE2* gene product inactivates GS by the attachment of glutathione, perhaps to the sulfur of a cysteine residue. We have purified the URE2 protein (unpublished result) and are engaged in exploring this possibility; however we have not been able to detect glutathione *S*-transferase activity (unpublished result).

The possible target of the *URE2* gene product in the regulation of gene expression may be the product of the *GLN3* gene. It has been shown that the ability of the cell to increase the level of GS, NAD-GDH, and other enzymes in response to replacement of glutamine by glutamate as source of nitrogen depends on the product of *GLN3* (40). We have confirmed these results by using true null alleles and have also shown an important difference in the response of *GLN1* and *GDH2* to the products of *GLN3* and *URE2*. Growth on glutamate is essentially as effective as a disruption of *URE2* in raising the level of *GLN1* expression, but in the case of *GDH2*, disruption of *URE2* is much more effective than growth on glutamate in increasing expression of this gene; nonetheless, this disrupted strain can still respond to the nitrogen source. Furthermore, experiments with diploids have shown that in the case of the regulation of *GDH2*, the dosage of *GLN3* plays an important role; reducing the dosage of *GLN3* by one-half relative to the dosage of *GDH2* reduces the level of NAD-GDH by one-half in glutamate-grown cells with functional *URE2*. Moreover, in diploid cells lacking *URE2*, growth on glutamine considerably reduces the expression of *GDH2* when *GLN3* is provided in single dosage but not when it is provided in double dosage. These results are in accord with the view that the product of *GLN3*, an activator of the expression of *GLN1* and *GDH2*, can be inactivated by glutamine but that this inactivation is greatly stimulated by the product of *URE2*. We assume that in wild-type glutamate-grown cells, the *GLN3* gene product is kept partially in an inactive form by the *URE2* gene product and that the intracellular concentration of active *GLN3* gene product is sufficient for full activation of *GLN1* expression but not of *GDH2* expression. Disruption of *URE2* consequently increases the active *GLN3* gene product to a level sufficient for full activation of *GDH2* expression. Similarly, reducing the *GLN3* dosage relative to that of *GDH2* in glutamate-grown diploid cells with functional *URE2* results in decreased expression of *GDH2*.

The fact that the gene dosage of the *URE2* product does not affect the expression of *GDH2* and *GLN1* in diploid cells is compatible with the view that the interaction between the products of *GLN3* and *URE2* is not stoichiometric but rather that the product of *URE2* acts catalytically on the *GLN3* gene product. Unpublished results in our laboratory have shown that neither transcription nor the initiation of translation of *GLN3* is subject to regulation by glutamine and that substantial increase in the dosage of *GLN3* does not increase the expression of *GLN1* and *GDH2* in glutamine-grown cells as much as the disruption of *URE2* (35a). Similar experiments indicate that the level of URE2 protein is also not regulated by glutamine (unpublished result). Moreover, experiments with *URE2-lacZ* and *GLN3-lacZ* fusions indicate that there is more initiation of translation of *GLN3* than of *URE2*. Therefore, unless the product of the *GLN3* gene is much more unstable than that of the *URE2* gene, the intracellular concentration of the *GLN3* gene product should

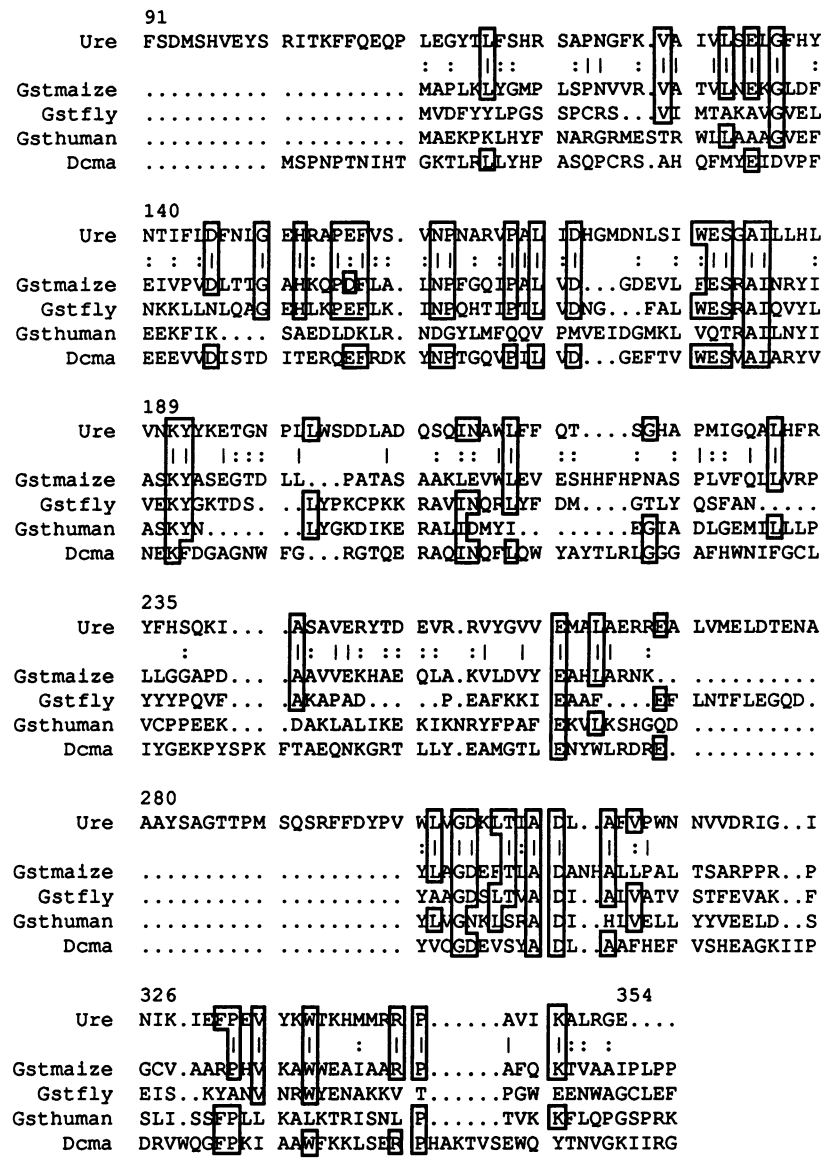

FIG. 6. Sequence comparison of URE2 with glutathione *S*-transferases. Amino acid sequences of URE2, maize glutathione *S*-transferase III (19), *Drosophila* glutathione *S*-transferase 1-1 (54), human glutathione *S*-transferase 2 (2), and *Methylobacterium* sp. strain DM4 dichloromethane dehalogenase (28) are compared. Boxes indicate residues that are identical in URE2 and at least two of the other enzymes. A direct comparison between URE2 and maize glutathione *S*-transferase is also included. Identities are indicated with a vertical line; conserved matches (neutral weak hydrophobic: P, A, G, S, T; hydrophilic acidic: Q, N, E, D; hydrophilic basic: H, K, R; hydrophobic: L, I, V, M; hydrophobic aromatic: F, Y, W) (11) are indicated with a colon. Numbering is according to the sequence of URE2.

exceed that of the *URE2* gene product. This supports the idea that the *URE2* gene product acts catalytically on the *GLN3* gene product; it is a formal possibility that other gene products are involved which interact with GLN3 or URE2, but there is no evidence to support this idea.

The hypothesis that glutamine causes the *URE2* gene product to inactivate GS and the GLN3 protein raises the question of the manner in which the inactive proteins are reactivated in response to shifting glutamine-grown cells to a medium with glutamate as a source of nitrogen. It has been shown that this reactivation occurs rapidly (39), but we do not know whether the product of *URE2*, alone or even in combination with other proteins, is responsible for this reactivation.

To better understand the roles of *GLN3* and *URE2*, it might be useful to examine nitrogen regulation in other organisms. The effects of *GLN3* and of *URE2* in *S. cerevisiae* have their counterparts in the effects of the products of *nit2* (a positive regulator for use of less favored nitrogen sources) (16) and of *nmr* (a negative regulator for use of less favored nitrogen sources) in *Neurospora crassa* (55). There is significant homology between *GLN3* and *nit2* (35a) but no striking homology between *URE2* and *nmr*.

The fact that the *URE2* gene product is involved in the regulation of both *GLN1* expression and GS activity has its counterpart in enteric bacteria. The product of the *glnB* gene, P_{II}, is involved in the repression of *glnA* (which codes for GS) by glutamine and in the inactivation of GS, but P_{II}

works through other proteins to carry out these functions (for a review, see reference 31). One might suppose that the *URE2* gene product requires other proteins to carry out its function and that mutations affecting these proteins would result in phenotypes similar to those resulting from mutations in *GLN3*. Such *gln3*-like mutants were isolated and found to fall into five complementation groups, but in all cases introduction of a *ure2* mutation into these backgrounds resulted in the phenotype characteristic of the *ure2* mutation. Apparently, mutations in these genes increase the ability of the *URE2* protein to prevent expression of *GDH2* and *GLN1* in glutamate-grown cells. This leaves open the question of how the *URE2* gene product inactivates GS and expression of *GLN1* and *GDH2*. Further analysis of the protein may yield the answer.

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